RADIATION EFFECTS ON RAT TESTES

V. STUDIES ON LYSOSOMAL ENZYMES (ACID PHOSPHATASE AND ACID DNase) AND THEIR PHYSIOLOGICAL SIGNIFICANCE FOLLOWING PARTIAL BODY GAMMA-IRRADIATION

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Summary. Acid phosphatase is present in the nucleus and cytoplasm of cells in the seminiferous tubules and the interstitium of rat testes. The effect of irradiation on acid phosphatase is dependent on the environmental temperature and the dose of irradiation. It appears that initial rise in the enzyme at a low radiation dose and a high environmental temperature or at a high dose and low temperature is associated with a lysosomal breakdown of the germinal cells of the testes. A decrease in acid phosphatase in the advanced stages of radiation injury is a secondary radiation effect which may lead to decreased metabolic synthesis of phosphate esters owing to the unavailability of orthophosphate in the testicular tubules. The reduced acid phosphatase activity can be detected in the seminiferous tubules, suggesting that the enzyme activity is related to the state of the germ cell population. An initial increase in acid phosphatase is matched by an initial rise in acid DNase within hours of irradiation, further suggesting that there is radiation interaction with the cells of the germinal epithelium. The enhanced activity of DNase following a 2nd week of irradiation at 2000 R confirms the phagocytic activity of the non-germinal cells.

INTRODUCTION

The study of testicular metabolic processes after ionising irradiation is complicated by difficulties in interpretation owing to the presence of a variety of different cells and the interplay of the pituitary (Steinberger, 1964). Though enzymatic patterns have often been related to changes in cell types during progressive stages of spermatogenesis (Males & Turkington, 1971), there are few studies on the metabolic and enzymatic pattern of irradiated testes (Steinberger & Nelson, 1957; Steinberger & Wagner, 1961; Pecora & Arate, 1964; Ito, 1966; Gupta & Bawa, 1970, 1971, 1974a, 1974b, 1975). The early effects of radiation injury are often related to a decrease in the content and synthesis of nucleic acids (Alekseeva & Timofeeva, 1959; Valenta & Kolousek, 1963; Sapsford, 1965; Gupta & Bawa, 1970, 1974b). Okada (1970) emphasized the rôle of ionic permeability and release of lysosomal enzymes in cell degeneration

following irradiation. The present observations concern the study of lysosomal enzymes in irradiated testes and suggest that the changes in these enzymes may be related to the radiation injury of the germinal epithelium and to the phagocytic activity of the non-germinal cells.

MATERIALS AND METHODS

Radiation procedure and treatment of tissues

Normal white albino male rats weighing 150 to 200 g and of a single strain were assigned to thirty-four groups, with four to six rats in each group. The rats were anaesthetized by intraperitoneal injections of pentobarbitone sodium (25 mg/kg body weight) and the body inferior to the penis of each rat was irradiated with a single surface dose of γ -radiation at a distance of 65 cm from a cobalt-60 source (Gupta & Bawa, 1974a) shielded in a Theratron (Canada) radiotherapy unit. The total surface dose of radiation varied from 720 to 6000 R. Four to six sham-irradiated rats acted as controls for each set of observations. During and after irradiation, the animals were normally maintained at a temperature of 30 to 40°C, but in one set of experiments the rats were kept at an environmental temperature of 12 to 25°C. For those rats observed over a period of 11 months, the temperature ranged between 12 and 40°C. Food was withdrawn 6 to 8 hr before the rats were killed by decapitation. The testes of the rats in each group were pooled and frozen. A randomized portion of parenchymal tissue was homogenized in a Potter-Elvehjem homogenizer to yield 5 to 10% aqueous homogenates. Whole homogenate was used for the assay of acid phosphatase but the supernatant fraction obtained after centrifugation at 3200 g was used for assay of acid DNase. The assay values for the enzymes of the rats in the control groups were pooled and their mean values with standard deviations were determined. This mean value of the enzyme was assumed to be the mean for normal rats. The relative values of the irradiated rats compared to those of the controls were also plotted separately for convenience of interpretation.

Separation of seminiferous tubules

Partial body irradiation of the testes with a total dose of 720 R was performed according to the method described above. Thirty days after irradiation, the seminiferous tubules of the testes of normal and irradiated rats were separated from the interstitium according to the method of Hall, Irby & de Kretser (1969) in Ringer bicarbonate buffer. When all the tubules were removed, the interstitial tissue in the remaining portion consisted of Leydig cells, connective tissue and blood vessels. Acid phosphatase activity was determined in homogenates of the two different portions.

Subcellular fractionation

Mitochondria from homogenates of whole testes were fractionated according to the method described by Clausen (1969) using 0.33 M-sucrose as the dispersion medium. The nuclear fraction separated at 900 g (Fraction I), the mitochondrial fraction separated at 10,000 g (Fraction II) and the remaining

cytosol with microsomes intact (Fraction III) was analysed separately for acid phosphatase activity.

Determination of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2)

A volume (1 ml) of β -glycerophosphate was hydrolysed by 0.5 ml tissue homogenate for 1 hr at 37°C and pH 5.6 (acetate buffer). The reaction was terminated by 0.5 ml of 10% trichloroacetic acid (TCA), and orthophosphate was determined in the supernatant. A unit of enzyme activity was defined as the amount which liberated 1 μ g orthophosphate in 1 hr at 37°C (Gupta & Bawa, 1970).

Determination of acid deoxyribonuclease

The activity of DNase II (deoxyribonucleate 3'-oligonucleotidohydrolase: EC 3.1.4.6) was determined by a modified method of Kurnick & Sandeen (1960). The concentrations of DNA as substrate, described in the original technique of Kurnick & Sandeen (1960), did not give appreciable activity of acid DNase. The concentrations of the substrate described in the present investigation were optimum concentrations for testicular DNase. The substrate mixture consisted of 2 ml aqueous DNA (8 mg/ml), 2 ml methyl green (0.4%), 4 ml acetate buffer (pH 4.6, 0.05 N), 2 ml magnesium chloride (0.64 M), distilled water and 0.02 ml aqueous sodium ethyl mercurithiosalicylate (1% merthiolate). The incubation medium contained 1.6 ml substrate mixture and 0.4 ml tissue homogenate. At 2 hr after incubation, 2 ml of a mixture of sodium citrate and 0.05 M-tris-buffer, pH 7.5 (1:3, v/v) was added. The mixture was stirred and allowed to stand in subdued light at room temperature overnight to assure complete fading off of free methyl green. The optical density was read at 640 nm. The unit of enzyme activity was defined as that which caused a reduction in the optical density of 1.0/min/ml reaction mixture at 37°C.

Other determinations

The methods of Lowry, Rosebrough, Farr & Randall (1951) and Fiske & Subbarow (1925) were used for the assays of protein and phosphorus respectively. Colour determinations were made in a spectronic-20 colorimeter spectrophotometer. Details regarding the number of animals and the analyses are given in the legends of the Text-figures.

RESULTS

Acid phosphatase

The normal mean value $\pm$ S.D. for acid β -glycerophosphatase in the testes was 133 ± 19 units/100 mg tissue and 35.2 ± 8.3 units/mg protein (Text-figs 1 and 2). An increase in the specific activity of the enzyme was demonstrable during the first week of irradiation at both lethal and sub-lethal doses (Text-fig. 2). Though this increase in the activity fell within the normal range for rat testes, it was significant ($P < 0.05$) when compared with the results of pooled analyses of the data for the sham-irradiated controls. After the first week, the

acid phosphatase activity decreased and became constant 10 to 24 days after irradiation (Text-fig. 2). The activity of the enzyme/testis decreased for 10 to 24 days, but maintained reasonably constant values thereafter (Text-fig. 2c). The effect of different doses of irradiation on acid phosphatase activity is shown in Text-fig. 3. The maximum damage/testis occurred at 2000 R. The activity/mg protein increased slightly between 4000 and 6000 R, but the increase in the enzyme activity/testis beyond 2000 R was apparently only slight.

TEXT-FIG. 1. Acid phosphatase activity expressed as (a) per 100 mg testis and (b) per mg protein, in the testis after partial body γ -irradiation of rats at different temperatures. Each point represents the mean for six irradiated rats and is based on three to five analyses. $\circ$, 720 R at $35 \pm 4^\circ\text{C}$ (range 30 to 40°C); $\bullet$, 2000 R at $35 \pm 4^\circ\text{C}$; $\triangle$, 2000 R at $18 \pm 6^\circ\text{C}$ (range 12 to 25°C). The hatched area represents the mean $\pm$ S.D. for normal rats.

The environmental conditions in which the animals were maintained seemed to modify the radiosensitivity of testicular acid phosphatase. At the lethal dose of 2000 R and the higher environmental temperature, the activity of the enzyme did not change over the first 5 days but subsequently declined within 10 days and maintained a constant value thereafter (Text-fig. 2). At a low environmental temperature, however, the rise in the activity within the first

TEXT-FIG. 2. Acid phosphatase activity, expressed as a % of the control values (a) per mg protein; (b) per 100 mg testis; (c) per testis, in the testis after partial body γ -irradiation of rats at different temperatures. Each point represents six irradiated and six control rats and is based on three to five analyses. $\circ$, 720 R at $35 \pm 4^\circ\text{C}$; $\bullet$, 2000 R at $35 \pm 4^\circ\text{C}$; Δ , 2000 R at $18 \pm 6^\circ\text{C}$.

TEXT-FIG. 3. Acid phosphatase activity expressed as a % of the control values, in the rat testis after various doses of partial body γ -irradiation. Each point represents six irradiated and six control rats and is based on six analyses. $\circ$, Units/mg protein; $\bullet$, units/mg tissue; Δ , units/testis.

week was followed by a decline within 24 days. A similar decline after an initial rise in acid phosphatase activity was observed at a dose of 720 R but at a higher environmental temperature.

On subcellular fractionation, the acid phosphatase activity was associated mainly with Fractions I and III which were rich in nuclear and microsomal contents respectively. Little activity was associated with Fraction II which mainly contained mitochondria (Table 1). Irradiation produced different types of response from the microsomal and nuclear enzymes depending on the dose and the time of irradiation, i.e. the activity of the nuclear enzyme declined whereas that of the microsomal enzyme was enhanced 5 days after irradiation at 2000 R. After irradiation at 720 R, the cytoplasmic acid phosphatase was reduced on the 30th day, at a time when irradiated interstitial cells showed enhanced activity (89.8 ± 14.0 units/mg protein; $P < 0.05$) and

Table 1. Subcellular distribution of acid phosphatase in control and irradiated rat testes

	Experiment 1			Experiment 2	
	720 R*	2000 R*	Sham-irradiated	720 R†	Sham-irradiated
Whole testis					
Units/100 mg tissue	139.0	122.0	113.0	86.2	121.2
Units/mg protein	28.9	28.0	25.1	25.5	33.0
Nuclear fraction (I)					
Units/100 mg tissue	10.0	34.6	39.1	18.0	35.5
Units/mg protein	9.0	27.6	31.7	28.1	43.2
Mitochondrial fraction (II)					
Units/100 mg tissue	11.0	11.5	8.7	2.2	4.5
Units/mg protein	16.0	13.3	12.6	6.8	15.0
Microsomal fraction (III)					
Units/100 mg tissue	117.4	152.6	117.0	91.4	201.0
Units/mg protein	48.3	62.7	48.1	55.7	121.0

* Killed 5 days after irradiation.

† Killed 30 days after irradiation.

seminiferous tubules gave a negative response (31.0 ± 5.1 units/mg protein; $P < 0.01$) compared to that of the controls (61.8 ± 12.0 units/mg protein and 76.0 ± 15.2 units/mg protein respectively).

Acid deoxyribonuclease II

The mean activity ($\pm$ S.D.) in the supernatant from centrifuged homogenates of normal rat testes was 0.195 ± 0.03 units/100 mg fresh tissue and 0.042 ± 0.004 units/mg protein (Text-fig. 4). As early as 4 to 6 hr after irradiation, the activity in the fresh tissue supernatants was 0.27 units in rats irradiated at 720 R compared to 0.20 units in the control rats. The difference in the activity of the enzyme in the fresh supernatants of testes from control and irradiated rats was more than twice the standard deviation ($P < 0.05$). After an initial increase within 4 to 6 hr at 720 R and 16 hr at 2000 R, acid DNase activity tended to be normal on Days 4 and 7, respectively, at lethal and sub-lethal

doses. Thereafter, following an increase, the activity continued to show a similar pattern over a period of 30 days and 11 months (Text-fig. 5). During subsequent periods, the over-all increase per mg testis and per mg protein at 2000 R was well-marked for those rats subjected to 720 R.

TEXT-FIG. 4. The activity of acid deoxyribonuclease in the testis of control rats (open columns) and of rats at different times after (a) 2000 R and (b) 720 R (hatched columns). Each column represents the mean for six irradiated and four controls rats and is based on four analyses.

DISCUSSION

Acid phosphatase

Studies on the effects of whole body irradiation on lysosomal enzymes in lymphoid tissues have indicated that the primary cause of death of the cells after irradiation may be due to the release of hydrolytic enzymes from the damaged lysosomes (Okada, 1970). The increase in the activity of acid phosphatase during the first week of irradiation may, therefore, be due to lysosomal breakdown of testicular cells or to increased phagocytic activity of the Sertoli cells (Nebel, 1959; Hugon & Borgers, 1966). Blackshaw & Hamilton (1970) suggested that the initial lesion during heat treatment may affect the cell wall of late pachytene spermatocytes, leading to major ionic imbalance and release of lysosomal enzymes with subsequent dissolution of the cell. Other studies of the local effects of heat on the testis suggest loss of acid phosphatase from the

TEXT-FIG. 5. The activity of acid deoxyribonuclease expressed as a % of the control values in units/100 mg tissue (open columns), units/mg protein (solid columns) and units/testis (hatched columns) on different days after partial body γ -irradiation at (a) 720 R and (b) 2000 R. Each column represents the mean for six irradiated and four control rats and is based on four analyses.

tubules (Bawa, Kanwar & Singal, 1971) and an increased concentration in the interstitium (Waites & Ortavant, 1968; Blackshaw, 1970). It appears that the response of rat testicular acid phosphatase to radiation resembles the response to local hyperthermia in the seminiferous tubules (Bawa *et al.*, 1971) and interstitium (Waites & Ortavant, 1968).

Ellis & Berliner (1963) reported a triphasic response of androgen production (including testosterone) to radiation. It was observed that androgen production increased during the first week after total body irradiation of mice. Since it has been suggested that lysosomes are involved in steroid synthesis and are inhibitory to androgen production (Frank & Christensen, 1968; Dietert & Scallen, 1969; Russo, 1970), it is possible that the enhanced activity of lysosomal enzymes during the first week is in some way related to the androgen production.

The studies on teased seminiferous tubules, however, suggest that the depleting action of the enzyme resides in this part of the testis and in the isolated nuclei from the tissue homogenates from rats 30 days after irradiation (Table 1). In rat testes, acid phosphatase has been shown to be present in lysosomes of

Sertoli cells, spermatocytes and spermatozoa (Males & Turkington, 1971). The late response of testicular acid phosphatase to radiation appears to be due to the disappearance of late spermatocytes and spermatids (Gupta & Bawa, 1974c). Rappoport (1959) has reported decreased production of nucleoside esters in testes 24 hr after irradiation. Since acid phosphatase plays an important rôle in producing orthophosphate, it appears that the loss of acid phosphatase in the nuclear fraction of the testis is responsible for reduced synthesis of the nucleotide esters required in the synthesis of nucleic acids and in the differentiation of the germ cells. This suggests that nuclear and microsomal enzymes may be operative in damaging the germ cells through different mechanisms. The increase in the microsomal fraction can be related to the release of lytic enzyme due to fragility of the lysosomes in the cells of the tubules. Lack of enhanced enzymatic activity in the first week at the high environmental temperature and a dose of 2000 R was presumably due to the sensitizing action of temperature in radiation injury and to the sudden disappearance of spermatocytes and spermatids. The effect of temperature during and after irradiation is well documented. Many controversies regarding the radiosensitivity of the germ cells can thus be resolved if the total conditions of the experiment are stated.

Acid deoxyribonuclease

The high requirement of substrate by testicular DNase suggests that the enzyme has a high K_m value and forms an unstable enzyme-substrate complex. Though acid phosphatase activity continued to decline after the first week of irradiation, acid DNase exhibited an upward trend within hours of commencing irradiation. Levels tended to decrease to normal within 7 days and thereafter started to increase again. The levels by Day 4 after a dose of 2000 R and by Day 7 after 720 R presumably coincided with maximum damage to the germ cells. Once the germ cells were lost, the activity started to increase because of concentration effects of Sertoli and interstitial cells per unit mass of the testis. The subsequent increase in the activity of acid DNase seems to be distinctly related to phagocytic activity of non-germinal cells which predominate at a time when the seminiferous tubules are devoid of germ cells (Gupta & Bawa, 1974c, 1975). The phagocytic activity of testis may be due to an imbalance of electrolytes (Gupta & Bawa, 1974a) across the germinal and non-germinal cells of the seminiferous tubules. Lacy (1964) found that the effects of irradiation and oestrogens on the testis led to the accumulation of lipids in the Sertoli cells, originating perhaps from degenerating germ cells (Lacy & Lofts, 1961). Hugon & Borgers (1966) noted dissolution of spermatogonia within a few hours of irradiation and phagocytosis was accompanied by the appearance of lysosomes in the Sertoli cells which, at this stage, showed a strong acid phosphatase reaction. The observations of the present study on the stimulated activity of acid phosphatase and DNase during the first week and the increased activity of DNase subsequently are in agreement with the observations of Lacy & Lofts (1962) and Hugon & Borgers (1966). We have suggested that irradiation of rat testes is associated with hyperplasia of the interstitium and the accumulation of glycogen in the non-germinal cells though, in normal

testes, these cells could not be shown to contain enough glycogen (Gupta & Bawa, 1974c). It is possible that glycogen is acting as a reserve substrate for the phagocytic activity of Sertoli cells. According to Blackshaw & Hamilton (1970), however, increased activity of lysosomal enzymes with ionic imbalance is an indication of some defect of the late pachytene spermatocytes. A point of difference between the two lysosomal enzymes after the first week can be resolved if it is assumed that the late germ cells are richer in acid phosphatase and thus subsequent loss of acid phosphatase is primarily associated with the loss of the germinal cell population (Males & Turkington, 1971).

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